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Choosing a secondary antibody: A guide to fragment specificity

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Introduction

A. Definition of Primary and Secondary Antibodies

Immunodetection depends on specific binding interactions between antibodies and their antigens. Antibodies that have been produced to detect a target molecule are called primary antibodies (1° Ab) because they bind directly to the molecule of interest. If a primary antibody is labeled with a fluorescent tag or an enzyme capable of producing a visible signal upon addition of the appropriate substrate, then probing with a primary antibody allows direct detection of the target antigen. Alternatively, if the primary antibody is not labeled with a detectable tag, a secondary antibody (2° Ab) with a detectable tag may be used. An antibody whose antigen is another antibody is called a secondary antibody; it binds to a primary antibody, allowing indirect (secondary) detection of the primary antibody's antigen.

Using secondary antibodies provides several advantages over direct detection with labeled primary antibodies. Most importantly, primary antibodies are typically expensive to buy or produce, and researchers may not wish to risk poor recovery or complete inactivation by subjecting the antibody to a labeling procedure to make it detectable. In addition, using an appropriate secondary antibody can provide signal amplification by multiplying the number of label molecules that can be bound per target molecule.

B. Polyclonal Characteristics of Secondary Antibodies

Primary antibodies are frequently produced as monoclonal antibodies. By contrast, most commercially-available secondary antibodies are polyclonal, being purified directly from the serum of animals that had been immunized with a particular antibody species, subclass and fragment type. The specificity of a secondary antibody depends on the antibody sample that is injected as the immunogen and on the type of purification used to prepare the final product.

As an example, consider a goat that is immunized with whole mouse IgG (all subclasses). A variety of goat antibodies will be produced that bind to different sites on the subclasses of mouse IgG that were injected. In addition, some of the many goat antibodies in the harvested serum may cross-react to other mouse immunoglobulins besides IgG; some may even cross-react to other species (human, rat, rabbit) of IgG. A more specific secondary antibody sample is obtained by purifying the subset of goat serum antibodies that bind to mouse IgG and by removing the subset that binds to other mouse immunoglobulin classes and other species of IgG. Commercial secondary antibodies are usually affinity-purified using the antigen (target antibody species), and many are also available that have been further purified by pre-adsorption to eliminate cross-reaction to other species of antibody.

C. Available Specificities and Forms of Secondary Antibodies

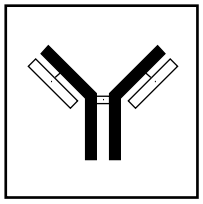
For certain applications, best results are obtained when antibody fragments are used as the primary antibody, in which case it may be beneficial to use a secondary antibody that is specific to that fragment. For example, in immunohistochemistry (IHC), signal-to-noise ratio may be enhanced if a Fab fragment of the primary antibody is used instead of the whole antibody molecule. The relatively smaller Fab fragment can more easily penetrate tissues to bind the target antigen. Using a Fab-specific secondary antibody in this case can reduce background signal that might otherwise result from binding to endogenous Fc molecules in the sample. To aid tissue penetration during the secondary antibody step, one might also use a secondary antibody that is an immunoglobulin fragment [e.g., Fab or $F(ab')_2$].

Thermo Fisher Scientific offers Pierce secondary antibodies that are specific for commonly-used primary antibody species (human, mouse, rabbit, goat, rat, etc.). For each target species, varieties of secondary antibody are available for particular immunoglobulin classes and fragment types. Some will bind all parts of whole IgG (heavy and light chains, H+L), or only the Fab or Fc region, or only the γ (gamma) chain. Still others are specific for IgM heavy chains (μ or Fc5μ), or the λ (lambda) or κ (kappa) light chains common to all immunoglobulins (IgG, IgA, IgD, IgE, and IgM).

The following is an explanation of whole and fragmented IgG and IgM. The advantages and disadvantages of using an antibody fragment instead of the whole molecule are further discussed. In all diagrams, the heavy chains are bold and the light chains are not.

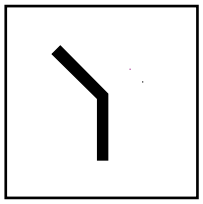
IgG Fragments

A. IgG Whole Molecule



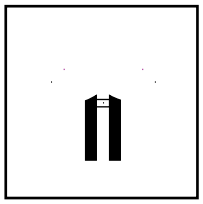
Whole IgG is a Y-shaped molecule composed of two heavy chains (~50 kDa each) and two light chains (~25 kDa each) held together by disulfide bonds and hydrophobic interactions. Anti-IgG 2° Abs are generated by injecting an animal (e.g., goat) with whole IgG from the species (e.g., mouse) that produced the 1° Ab. The resulting polyclonal 2°Ab recognizes both heavy and light (H + L) chains, allowing it to interact with the largest number of 1°Ab epitopes to produce the greatest possible signal. However, because light chains are shared by all immunoglobulin classes, using a 2° Ab that recognizes both heavy and light chains may result in cross-reactivity to other immunoglobulins. The problem is common in IHC applications because extraneous immunoglobulins are more likely to be present in heterogeneous tissue than in cell culture samples.

B. Gamma Chain of IgG



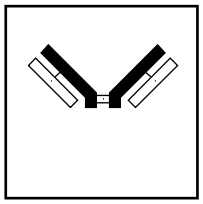
Gamma (γ) chains are the heavy chains that define the IgG class of immunoglobulins. (The "G" in IgG stands for gamma.) The γ chain contains only a portion of a single antigen binding site. The upper "arm" consists of a constant region and a terminal variable region. The variable region is involved in antigen recognition, allowing the same immunoglobulin type to generate specificity for many different antigens. Gamma-chain specific 2° Abs recognize all IgG fragments that contain all or part of a heavy chain: whole IgG, Fab, $F(ab')_2$, and Fc fragments. Gamma-chain specific 2° Abs are produced by injecting whole IgG into a host, affinity-purifying the Ab, and then adsorbing it to remove any light chain-reactive antibodies. Consequently, γ-chain specific 2° Abs will not cross-react with other immunoglobulins by binding to their light chains. These 2° Abs typically produce less nonspecific binding and background signal than 2° Abs for whole IgG (H + L).

C. Fc Fragment of IgG



The Fc (crystallizing fragment) of IgG consists of about one half of a pair of heavy chains. (It appears as the base of the Y-shaped whole molecule.) The upper portions of the heavy chains and all light chains are absent; hence an Fc fragment cannot bind antigen. Anti-Fc 2°Abs are produced by injecting a host with Fc fragments from another species that produced the 1°Ab. These Fc-specific 2°Abs will not cross-react with other immunoglobulin classes by binding to their light chains. Neither will they cross-react with non-IgG heavy chains. Because absence of cross-reactivity results in less background signal, anti-Fc 2°Abs are often preferred to those that recognize either whole IgG or the full γ chain.

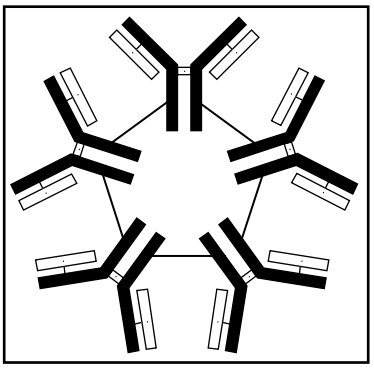
D. $F(ab')_2$ Fragment of IgG



The $F(ab')_2$ fragment of IgG consists of the whole IgG minus most of the Fc. (It corresponds to the arms of the Y-shaped IgG, held together at their junction, or hinge region.) $F(ab')_2$ fragments are useful because they are smaller than whole IgG molecules (100 vs 150 kDa, respectively), which enables better penetration into tissue samples, thereby facilitating better antigen recognition and signal generation in IHC applications. (Although Fab fragments are half the size of $F(ab')_2$ fragments, they cannot both bind and precipitate antigen because they have only one binding site.) As mentioned in the introduction, $F(ab')_2$ fragments can be produced from both 1° and 2°Abs. Anti-$F(ab')_2$ 2°Abs recognize the entire IgG light chain but only the "upper" portion of the heavy chain; they do not recognize the "lower," Fc region. Pierce Anti-$F(ab')_2$ antibodies are generated by injecting a host with the fragment (i.e., not by injecting whole IgG and then pre-adsorbing to remove those that bind Fc).

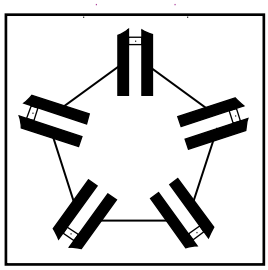
IgM Fragments

A. IgM Whole Molecule



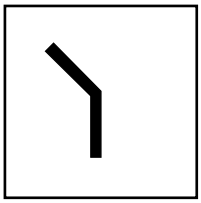
Whole IgM comprises five Y-shaped units connected through their Fc domains by a J-chain. The pentamer has ten heavy chains, ten light chains, and ten antigen-binding sites. Anti-IgM 2°Abs are generated by injecting a host with whole IgM. The use of 2°Abs that recognize whole IgM frequently results in unacceptable background and lower specificity for two reasons. First, light chains are shared by all immunoglobulin types. Second, IgG tends to be the predominant species in serum and other samples. Consequently, an Ab to whole IgM tends to cross-react with IgG light chains. Quite often a better choice is a 2° Ab that reacts specifically with the unique IgM mu (μ) heavy chain or the IgM pentameric Fc5μ fragment.

B. Fc5μ Fragment of IgM



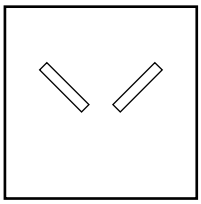
The Fc5μ fragment consists of the interconnected bases of the five Y-shaped units. The upper portions of the heavy chains (μ chains) and the entire light chains are absent. Pierce Anti-Fc5μ 2°Abs are generated by injecting a host with the fragment. Because these 2° Abs recognize the IgM fragment but neither immunoglobulin light chains nor non-IgM heavy chains, their use typically results in less nonspecific binding and background signal than a 2° Ab that recognizes whole IgM molecule.

C. Mu Chain of IgM



Mu (μ) chains are the heavy chains that define the IgM class of immunoglobulins. (The "M" in IgM stands for μ.) Individual μ chains are univalent with only a portion of a single antigen-binding site instead of ten antigen-binding sites, as on the whole pentamer. The light chains and "J" chain are absent. Mu-chain specific 2° Abs are produced by injecting a host with whole IgM and then adsorbing the anti-serum to remove antibodies against light chains. These 2°Abs can detect whole IgM, Fab, $F(ab')_2$, Fc5μ, and Fcμ fragments, as well as the μ chain itself. Because they only recognize epitopes found on the μ chain, cross-reactivity with other immunoglobulin light chains and non-IgM heavy chains is eliminated. Mu-chain specific 2° Abs typically produce less nonspecific binding and background signal than antibodies produced against whole IgM.

Light Chains of Immunoglobulins



Secondary antibodies are available to specifically recognize either the kappa (κ) or lambda (λ) light chains of human immunoglobulins. Light chains consist of constant and variable domains, each containing about 110 amino acids. The proximal constant domain of light chains is shared by all immunoglobulins (IgG, IgM, IgA, IgE, and IgD) within a species. The terminal variable domain is involved in antigen recognition. The two types of constant regions give the light chains their designation as either kappa or lambda. Whether kappa or lambda, all light chains are bound to heavy chains through disulfide bonds and hydrophobic interactions. Knowing the type of light chain that predominates in a sample can be critical. For example, lambda chains (and some kappa subgroups) do not bind well to Protein L. Consequently, Protein L is a poor choice for immunoaffinity purification of immunoglobulins whose light chains are of the lambda variety.

Summary

There are a number of reasons for using a labeled secondary antibody instead of a labeled primary antibody. Understanding the structure and specificity requirements of the intended assay system is essential to choosing an appropriate secondary antibody and obtaining the best possible results. When samples contain few sources of cross-reactivity, choosing a 2° Ab that recognizes a whole immunoglobulin molecule generally will provide the most signal. For samples containing endogenous immunoglobulins, using a 2° Ab that is fragment-specific to the primary antibody will provide the most specific signal. Still other applications, such as IHC, produce the best result when the specific 2° Ab is itself a fragment.

Thermo Fisher Scientific offers secondary antibodies that are specific for the commonly-used primary antibodies and fragments of different species that are discussed in this Tech Tip. Secondary antibodies are available without labels and with biotin, horseradish peroxidase, alkaline phosphatase or various fluorophore labels. Please refer to the Secondary Antibody Selection Guide for Pierce Protein Research Products at the company website for more help in choosing a secondary antibody.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.